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EIGHTH ANNUAL REPORT

HUMAN PLATELET SENESCENCE

ANNUAL SUMMARY REPORT

JULY, 1978

Simon Karpatkin, M.D.

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21701

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heterogeneity of monoamine oxidase
microtubules and aggregation

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

1. Evidence that large-heavy platelets (megathrombocytes) are released early from the bone marrow and become smaller-lighter platelets with time under basal conditions - and;
2. Heterogeneity of platelet protein content as determined by SDS-polyacrylamide gel electrophoresis of cell sap proteins of various platelet populations isolated by differential centrifugation in plasma - and;
3. Heterogeneity of platelet monoamine oxidase activity: correlation with platelet protein density - and;

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ABSTRACT (continued)

4. Evidence that megathrombocyte number determines platelet function and that significant numbers of megathrombocytes are routinely discarded by conventional methods of preparation of platelet-rich plasma; and
5. Evidence that intact microtubules are required for platelet aggregation.

For the past year our laboratory has been engaged in 4 areas of platelet (and megathrombocyte) research with regard to heterogeneity, production, mobilization and function. We have also been interested in platelet microtubules (tubulin).

1. Evidence that large-heavy platelets (megathrombocytes) are released early from the bone marrow and become smaller-lighter platelets with time under basal conditions.

2. Heterogeneity of platelet protein content as determined by SDS-polyacrylamide gel electrophoresis of cell sap proteins of various platelet populations isolated by differential centrifugation in plasma.

3. Heterogeneity of platelet monamine oxidase activity: correlation with platelet protein density.

4. Evidence that megathrombocyte number determines platelet function and that significant numbers of megathrombocytes are routinely discarded by conventional methods of preparation of platelet-rich plasma.

5. Evidence that intact microtubules are required for platelet aggregation.

SUMMARY OF PROJECTS

1. Evidence that large-heavy platelets (megathrombocytes) are released early from the bone marrow and become smaller-lighter platelets with time under basal conditions.

Rabbits were injected intravenously with the cohort platelet label, Se^{75}-selenomethionine. Blood was withdrawn on days 0 through 7, and the platelet-rich plasma separated into 5 different platelet density fractions by repetitively centrifuging the same sample of platelet-rich plasma at increasing gravitational force, (Figure 1). The heaviest platelet sediment fraction was enriched with larger platelets: mode, 2.90 fl; mean, 3.33 fl; megathrombocyte index, 8.45 fl. The lightest platelet sediment fraction was enriched with smaller platelets: mode, 2.02 fl; mean, 2.26 fl; megathrombocyte index, 5.63 fl (Table 1). Incorporation of isotope into the heaviest platelet fraction (specific activity, cpm/platelet) was considerably greater than incorporation into the lighter platelet fractions on day 1. The ratio of the heaviest platelet fraction specific activity to the specific activities of the platelets sedimented at higher g forces (lighter-smaller platelets) generally decreased on each day for 4 to 6 days (Figure 2, Table 2). Whereas incorporation of isotope into the heaviest platelet fraction declined on days 3 to 4, incorporation into the lightest 3 fractions remained the same. Cohort platelet survival curves revealed a significant lag period of 1-2 days for the incorporation of isotope into the lightest 3 platelet fractions compared to the heaviest 2 platelet fractions (Figure 3). The mean platelet survival of the lightest 2 fractions (3.1 and 2.1 days) was significantly shorter than the mean platelet survival of the heaviest 3 fractions (3.8, 4.0, and 4.1 days, respectively).

The data are compatible with two models: 1)Heavy-large platelets are, on average, young platelets which become lighter-smaller platelets with time and lose platelet membrane and/or cell sap components with time. 2) Heavy-large platelets and light-small platelets are produced independently by specific megakaryocytes. The heavy-large platelets incorporate more isotope than lighter-smaller platelets (possibly because of their megakaryocyte precursor). However, they are released earlier into the circulation than lighter-smaller platelets and are therefore younger platelets. The lighter-smaller platelets which are released later into the circulation have a shorter platelet survival.

2. Heterogeneity of platelet protein content as determined by SDS-polyacrylamide gel electrophoresis of cell sap proteins of various platelet populations isolated by differential centrifugation in plasma.

The cell sap of the 5 platelet populations was isolated following sonication and centrifugation at 100,000 g for 1 hour. Aliquots were reduced with dithiothreitol and electrophoresed on SDS-5% polyacrylamide gels. The protein was stained with Coomassie blue which generally revealed 10 prominent protein bands of molecular weight: (251,000), (218,000? myosin), (202,000), (142,000), (126,000), (104,000), (91,000), (66,000? albumin), (55,000? tubulin) and (46,000? actin). However, the lightest platelet fraction which comprised approximately 1% of the harvested platelet population had 6 absent to markedly diminished platelet proteins of molecular weight: (142,000), (126,000), (104,000), (91,000), (55,000? tubulin) and (46,000? actin).

Kinetic data from cohort and isotope labelling suggested that this light platelet fraction represented older platelets which had transmuted from heavier-larger platelets. The loss of specific platelet proteins would be compatible with a change in platelet volume and density which could lead to or be associated with platelet death.

3. Heterogeneity of platelet monamine oxidase activity: correlation with platelet protein density.

Platelet monamine oxidase activity (MAO) was examined in platelets from patients with Autoimmune Thrombocytopenic Purpura (ATP), (a condition characterized by low platelet counts and large platelets, secondary to increased peripheral destruction of platelets) and in platelets from patients with Reactive Thrombocytosis (RT), (a condition characterized by high platelet count and smaller platelets, secondary to increased platelet production). ATP patients had 2.3-fold lower platelet protein density (platelet protein in gm x 10^{-15}/unit volume of platelets (mean platelet volume in μ^3 x platelet count)) and 2.1-fold lower specific activity of MAO (using either benzylamine or tryptamine as substrate) than a normal group of subjects. On the other hand, RT patients had 1.5-fold higher platelet protein density and 1.5-fold higher S.A. of MAO than the normal group. This relationship of platelet protein density to enzyme specific activity was further investigated by examining MAO activity in the 5 different platelet subpopulations of normal subjects. Platelet MAO and protein content were heterogeneously dispersed with a 16-fold greater enzyme activity and 24-fold greater protein content in extreme heavy (enriched with large platelets) compared to extreme light platelet fractions (enriched with small platelets). When data from this differential centrifugation study in normal subjects, as well as data from the two aberrant groups are considered, it appears that high MAO activity

is associated with heavy platelets, densely packed with protein rather than with large platelets per se.

4. Evidence that megathrombocyte number determines platelet function and that significant numbers of megathrombocytes are routinely discarded by conventional methods of preparation of platelet-rich plasma.

Platelet aggregation velocity was measured in platelet-rich plasma with an aggregometer following addition of ADP, collagen or epinephrine. Platelet volume parameters: mean, mode, megathrombocyte index (large platelets) were determined with a Coulter Model B Counter attached to a P-64 Channel Analyzer with electronic recorder and correlated with platelet count. A negative linear relationship was found between platelet count and platelet volume, $r = -0.53$, $P<0.001$ (Figure 4). Platelet aggregation velocity induced with collagen or epinephrine (primary wave) was directly proportional to platelet volume and correlated best with megathrombocyte index, $r = 0.59$, $P<0.001$ and $r = 0.53$, $P<0.02$, respectively. Platelet aggregation velocity with ADP correlated with megathrombocyte index, $r = 0.62$, $P<0.001$ and did not correlate with platelet mean or mode volume, $r = 0.27$, $P>0.1$, $r = 0.22$, $P>0.1$, respectively (Figure 5). Platelet volume distribution curves of residual non-aggregated platelets revealed relative absence of larger platelets (Figure 6). Thus platelet aggregation velocity is proportional to platelet volume, but correlates best with the number of large platelets or megathrombocyte index, particularly with ADP-induced platelet aggregation. Further experiments with rabbit blood anti-coagulated with EDTA revealed that megathrombocytes were preferentially lost during the routine preparation of platelets via differential centrifugation and that these were entrapped within the buffy coat (Figure 7, Table 3).

These data could help to explain the clinical observation that purpura is often absent in profound thrombocytopenia due to increased platelet turnover (where megathrombocytes are increased). It is suggested that the clinical strategy for treatment of platelet disorders be better directed towards evaluation of platelet volume and function rather than number. It is further suggested that the harvesting of platelets for platelet transfusion should include recovery of heavy-large platelets which are routinely discarded with routine methods of preparation.

5. Evidence that intact microtubules are required for platelet aggregation.

Colchicine and vincristine are known to disrupt microtubules and have previously been shown to inhibit platelet aggregation induced by ADP, epinephrine and collagen. The effect of these anti-tubular agents on platelet aggregation induced by the Ca++ Ionophore A23187, as well as the aggregating agents ADP, epinephrine and collagen were investigated. The Ca++ ionophore is known to release calcium from the sarcoplasmic reticulum of other tissues. Human blood was freshly drawn and anticoagulated in 0.38% sodium citrate solution. Platelet-rich plasma was incubated at 37°C for 8 minutes with 10^{-4}M colchicine or 10^{-5}M vinblastine and then induced with 1-5 μgm of ionophore. A significant decline in the velocity of aggregation was observed (40-75% of control, 30 experiments). The same applied for induction with ADP (10^{-5}M), epinephrine (10^{-5}M) or collagen.

Table I

PLATELET VOLUME DISTRIBUTION PARAMETERS*

RPM	% Platelets	Mode	Mean	Mega. Index
800 (196 g)	32.8±1.6	2.90±0.08	3.33±0.06	8.45±0.19
1200 (420 g)	37.8±1.1	2.76±0.06	3.10±0.06	7.43±0.19
1600 (728 g)	21.7±1.1	2.52±0.06	2.75±0.06	6.63±0.18
1800 (924 g)	5.8±0.5	2.26±0.06	2.56±0.05	5.98±0.19
2000 (1120 g)	1.9±0.2	2.02±0.08	2.26±0.09	5.63±0.19

*Five different platelet sediments were obtained after successive differential centrifugation of the same rabbit platelet-rich plasma at 800, 1200, 1600, 1800 and 2000 RPM in a Sorval RC-3 centrifuge at 4° for 20 minutes. The values in parentheses represent the g forces. The platelet sediments were resuspended in one ml of Ringer-2mM EDTA solution, pH 7.1 and assayed for platelet count and platelet volume distribution. The platelet volume distribution curve was measured for mode, mean and megathrombocyte index. The values given represent the average and S.E.M. of 66 experiments.

Colchicine and vinblastine were additive in their inhibitory effects. Dibutyrl cyclic AMP (10^{-4}M) also inhibited the velocity of platelet aggregation. However, its effect with colchicine or vinblastine was synergistic rather than additive suggesting a different mechanism of action. Heavy water, D_2O, has previously been shown to stabilize microtubules in other tissues. Addition of D_2O at 25-75% final concentration shortened the lag time (20-70%) before induction of aggregation by the ionophore and enhanced the velocity of aggregation when added to platelets inhibited by colchicine or vinblastine (30 experiments). Our data indicate that intact microtubules are required for platelet aggregation following relocalization of calcium by the ionophore. Disruption of microtubules leads to the inhibition of calcium-induced aggregation of platelets. The above results contradict the current concept that microtubules merely act as a cytoskeleton.

Table II

SPECIFIC ACTIVITY RATIOS OF 800 RPM PLATELET SEDIMENT FRACTIONS TO OTHER RPM PLATELET SEDIMENT FRACTIONS ON DAYS 1 THROUGH 7 FOLLOWING SE^{75}-SELENOMETHIONINE INJECTION.*

Day	1200	1600	1800	2000	N
1	2.24	4.96	9.63	-	(9)
2	1.28	1.99	2.74	10.3	(10)
3	1.40	1.74	2.06	2.94	(15)
4	1.13	1.41	1.30	2.30	(9)
5	1.10	1.31	2.06	-	(7)
6	0.79	1.38	1.37	-	(6)
7	1.61	2.03	2.45	5.51	(12)

*Forty-four microcuries of Se^{75}-selenomethionine was injected into eight rabbits for each experiment (1 hour sample not shown in Table). Each rabbit served as an experimental point in the kinetic analysis. Forty ml of whole blood was removed from one rabbit and the PRP harvested at 600 RPM for 20 minutes at 4°C. Platelet sediments were then obtained at increasing RPM from the original PRP, and processed for platelet count and radioactivity. The specific activity obtained from the 800 RPM sediments (cpm/platelet x 10^{-6}) was divided by the specific activities obtained at 1200, 1600, 1800 and 2000 RPM respectively, to obtain the ratios. N refers to number of rabbits employed for each time point.

Table III

EFFECT OF CENTRIFUGATION AND SUCCESSIVE WASHES IN THE PREPARATION OF PLATELET-RICH PLASMA ON PLATELET YIELD

RPM	Initial PRP Percent	1st Wash Percent	2nd Wash Percent	3rd Wash Percent	Total Percent
500* (69)	59.4	4.8	5.3	-	69.5
1000 (280)	61.2	15.0	2.4	-	78.6
1000	68.7	15.0	3.0	1.5	88.2
1000	55.4	8.1	1.0	0.4	64.9
1000	78.0	20.0	3.5	1.0	102.5
1000	34.6	30.7	9.6	3.8	78.7
1000	45.1	5.6	9.0	2.3	62.0
1250 (455)	49.3	25.3	6.8	2.0	83.4
1250	41.2	28.6	6.6	1.5	77.9
Avg	54.8	15.0	5.2	1.8	78.4

*Whole blood was anticoagulated with EDTA and centrifuged at 500 RPM for 20 minutes at 4°C in a calibrated testube. The volume of the PRP and the platelet count were determined. The remaining red blood cells, white blood cells and 'trapped' platelets were then resuspended in isoton to the same volume as the original whole blood and recentrifuged at 500 RPM for 20 minutes for the first wash. The second and third washes were similarly treated. The g force is given in parentheses.

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Legend to Figures

Figure 1. Platelet volume distribution curves from subpopulations obtained following centrifugation of rabbit platelet-rich plasma (PRP) successively at 800 RPM (196 g), 1200 RPM (420 g), 1600 RPM (728 g), 1800 RPM (924 g) and 2000 RPM (1120 g) respectively, for 20 minutes at 4°C. The respective platelet sediments were resuspended in one ml of human Ringer-2mM EDTA, pH 7.1. An aliquot of this suspension was utilized for platelet volume distribution analysis on a Coulter counter Model B attached to a P-64 Channel analyzer with electronic recorder. The data is representative of 66 different rabbits.

Figure 2. Specific activity of various platelet subpopulations obtained by differential centrifugation of rabbit PRP on days 0 through 7 following the intravenous injection of 44 microcuries of Se^{75}-selenomethionine. Specific activity is expressed as cpm/platelet x 10^{-6}. S.E.M. is given for the mean of all experiments including zero values. Interrupted bars refer to the mean of all experiments excluding the zero values. The fraction over some of the bars refers to the number of zero value experiments divided by the total number of experiments.

Figure 3. Cohort Se^{75}-selenomethionine platelet survival curves obtained from the various platelet subpopulations isolated as in Figures 1 and 2 following injection of 44 microcuries of isotope. Platelet survival was determined from the time interval between the 50% ascending limb and 50% descending limb of the cohort curve (interrupted horizontal line). Platelet survival curves for the 800, 1200, 1600, 1800 and 2000 RPM fractions were: 3.8, 4.0, 4.1, 3.1 and 2.1 days respectively.

Figure 4. Correlation between whole blood platelet count and megathrombocyte index. The regression line was determined by the method of least squares and is given by the formula $y = 4.80x + 508$. The correlation coefficient, $r = -0.53$ has a P value less than 0.001.

Figure 5. Correlation between maximum average aggregation velocity and megathrombocyte index. A. ADP-induced aggregation (maximum average of 2.6×10^{-5}M and 2.6×10^{-6}M). Formula for regression line: $y = 34.74x$. The correlation coefficient $r = 0.62$ has a P value less than 0.001. B. Collagen-induced aggregation (maximum average of 1:18,000 and 1:36,000 dilution). Formula for regression line: $y = 30.24x$. The correlation coefficient $r = 0.59$ has a P value less than 0.001. C. Epinephrine-induced primary wave aggregation (maximum average of 5×10^{-5}M and 5×10^{-6}M). Formula for regression line: $y = 6.26x$. The correlation coefficient $r = 0.53$ has a P value less than 0.02.

Figure 6. Platelet volume distribution curves obtained from platelet-rich plasma before (——) and after (---) addition of collagen at a 1:36,000 dilution.

Figure 7. Effect of centrifugation and successive washes in the preparation of PRP on the platelet volume distribution curve. The platelet volume distribution curve of initial PRP (——) and that of residual platelets 'trapped' in the remaining buffy coat and red blood cell mass are compared after the first wash, (——), second wash (---) and third wash (···) of the remaining blood cells.

Recent Work Supported by Army Contract

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Fig 4.

PLATELET VOLUME DISTRIBUTION CURVES OF VARIOUS PLATELET FRACTIONS

1 WINDOW = 0.25 fl

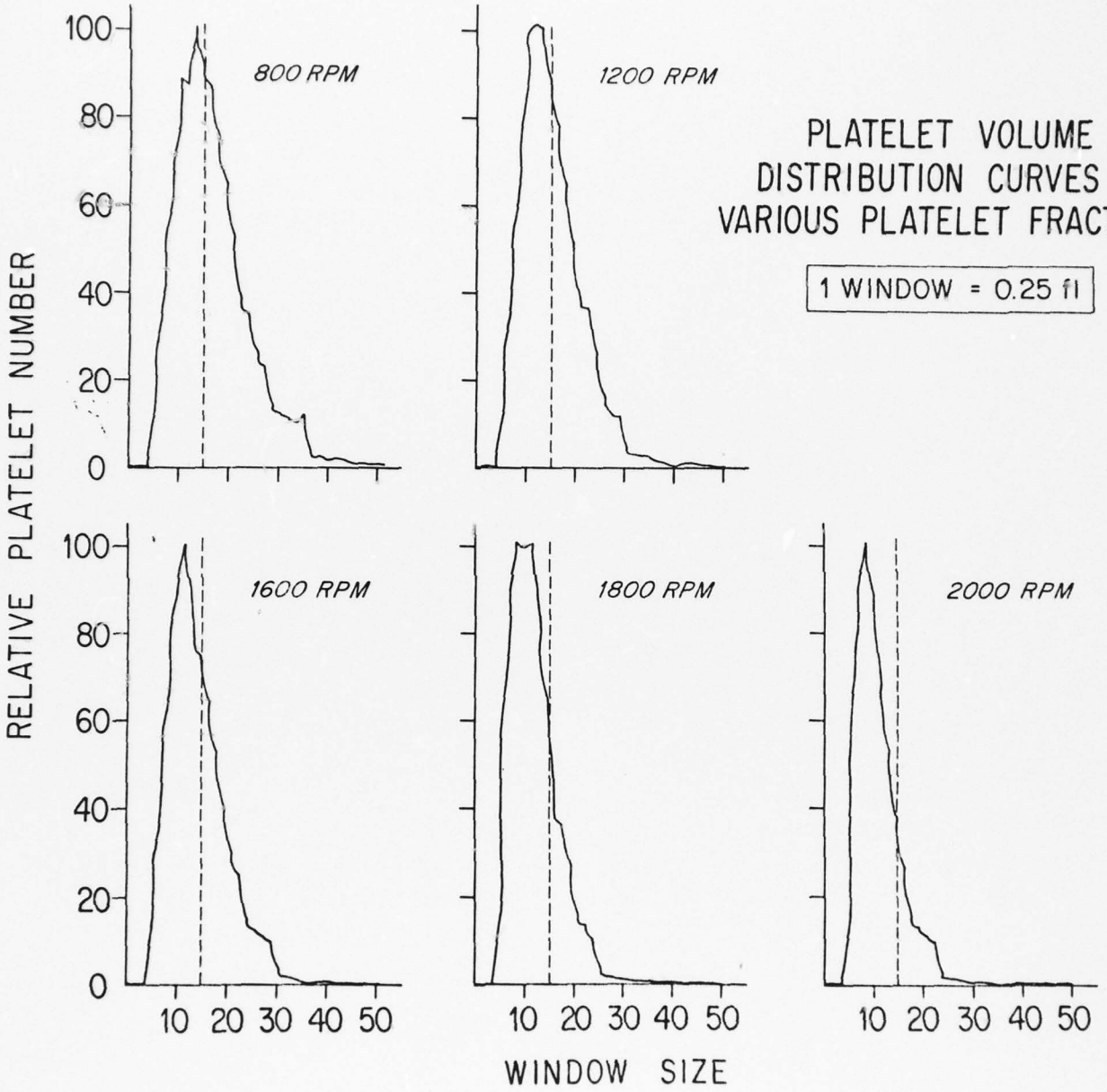


Fig 1.

Fig 2.

INCORPORATION OF Se^{75} - SELENOMETHIONINE INTO VARIOUS PLATELET FRACTIONS

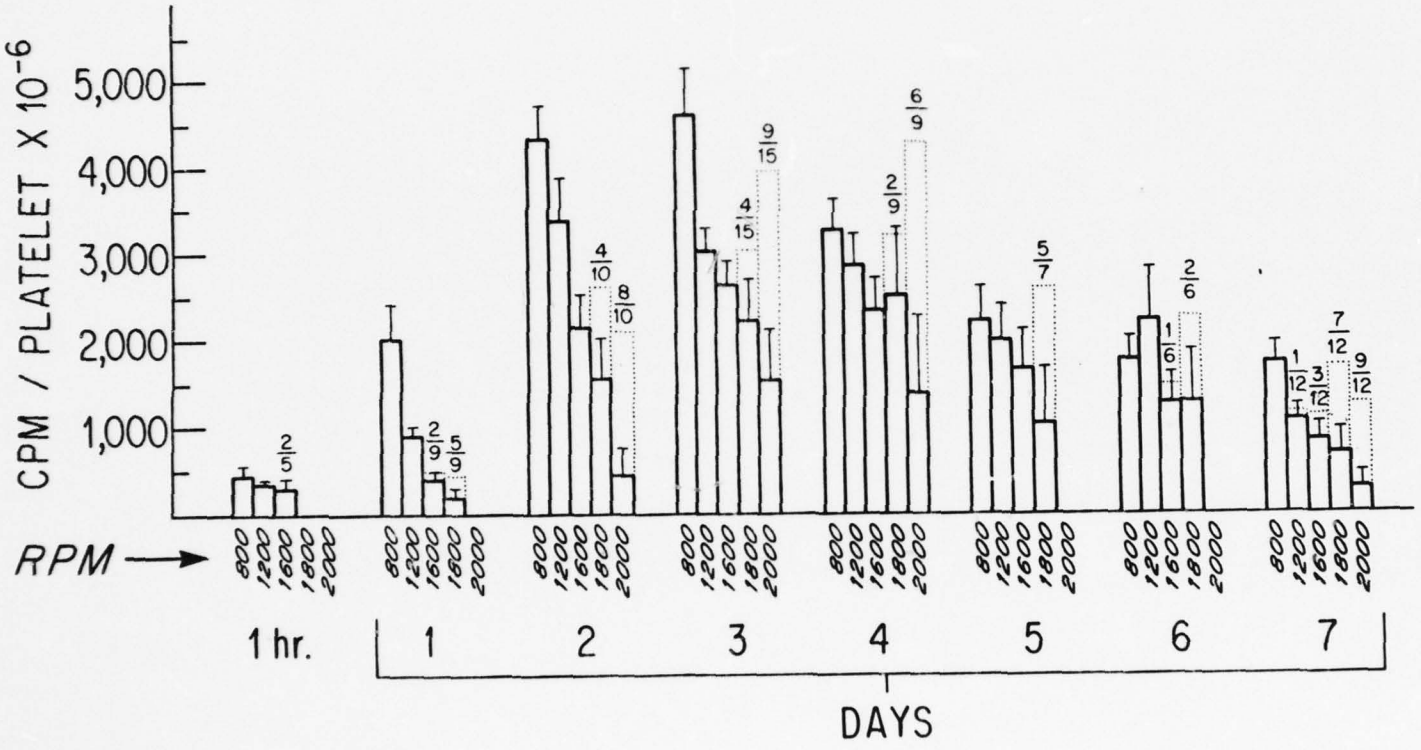


Fig 3

Fig 3.

Se^{75} - SELENOMETHIONINE COHORT SURVIVAL CURVES

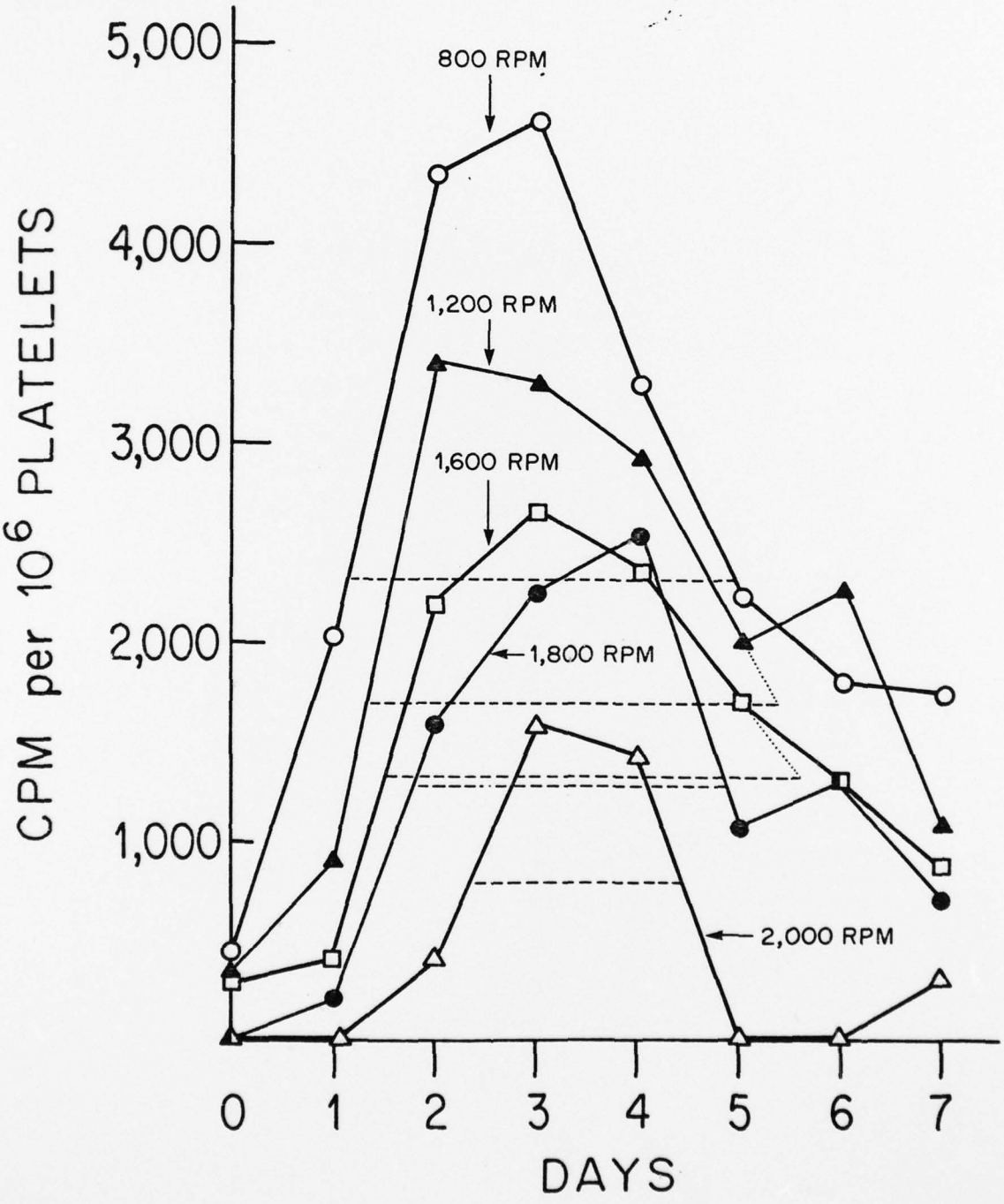
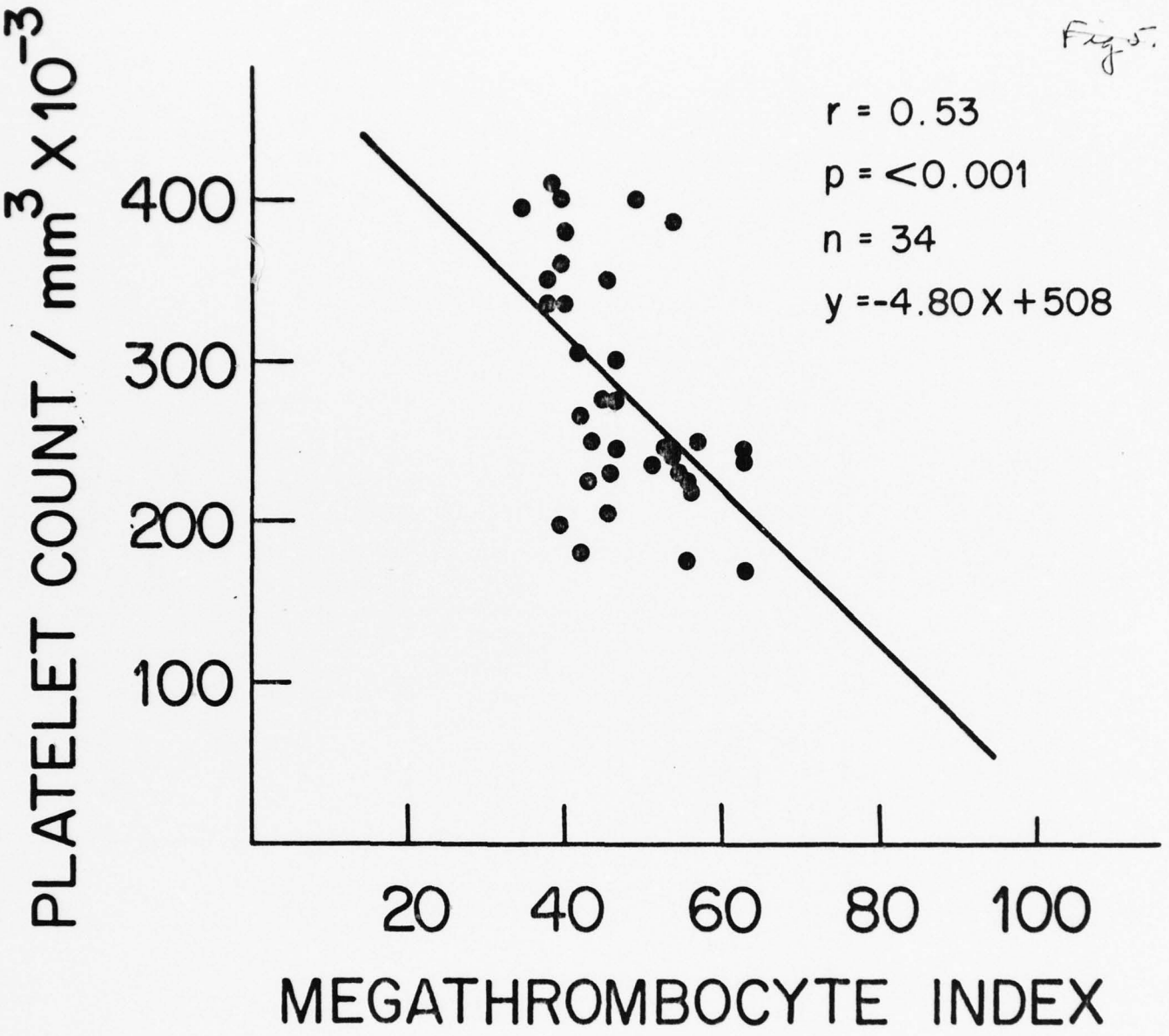
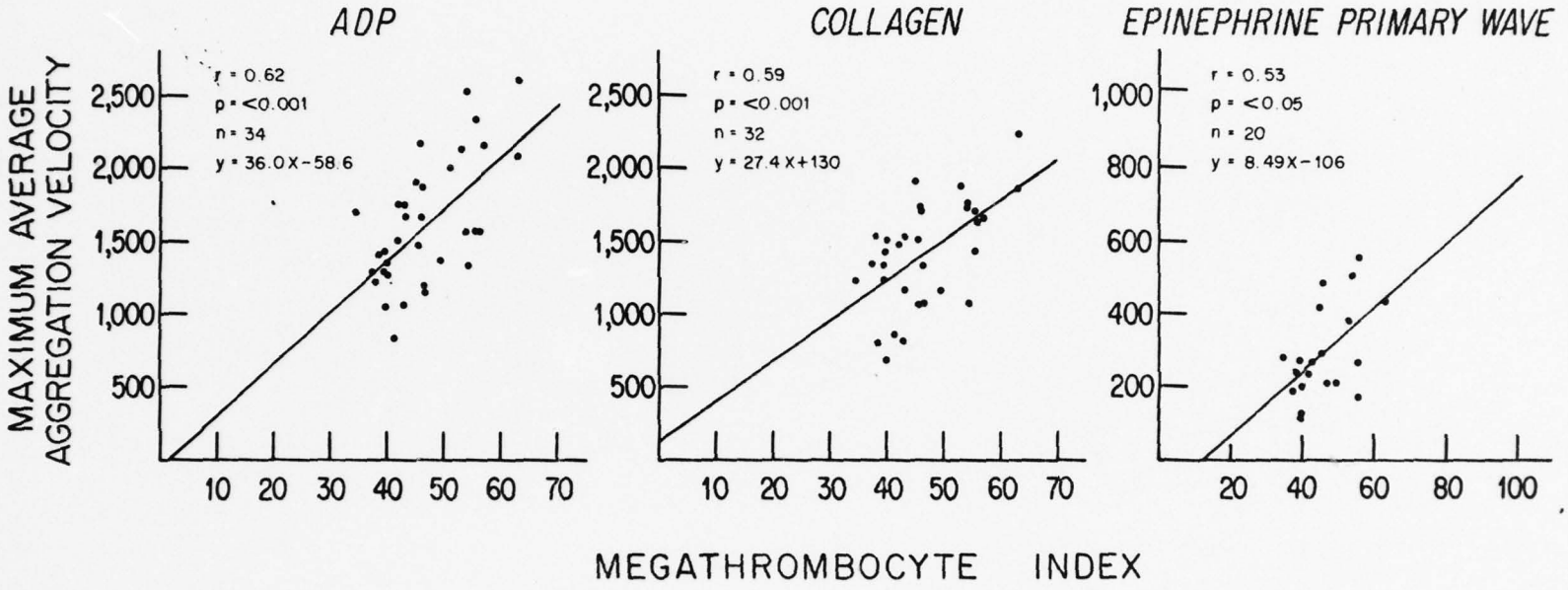


Fig 5.


PLATELET COUNT / mm^3 X 10^{-3}
400
300
200
100
r = 0.53
p = <0.001
n = 34
y = -4.80 X + 508
20
40
60
80
100
MEGATHROMBOCYTE INDEX

4 Fig

Fig 6.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COLLAGEN
EPINEPHRINE PRIMARY WAVE
MAXIMUM AVERAGE AGGREGATION VELOCITY
r = 0.62
p = <0.001
n = 34
y = 36.0X-58.6
r = 0.59
p = <0.001
n = 32
y = 27.4X+130
r = 0.53
p = <0.05
n = 20
y = 8.49X-106
2,500
2,000
1,500
1,000
500
1,000
800
600
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20 40 60 80 100
MEGATHROMBOCYTE INDEX

Fig 5

Fig 6.

PLATELET VOLUME DISTRIBUTION

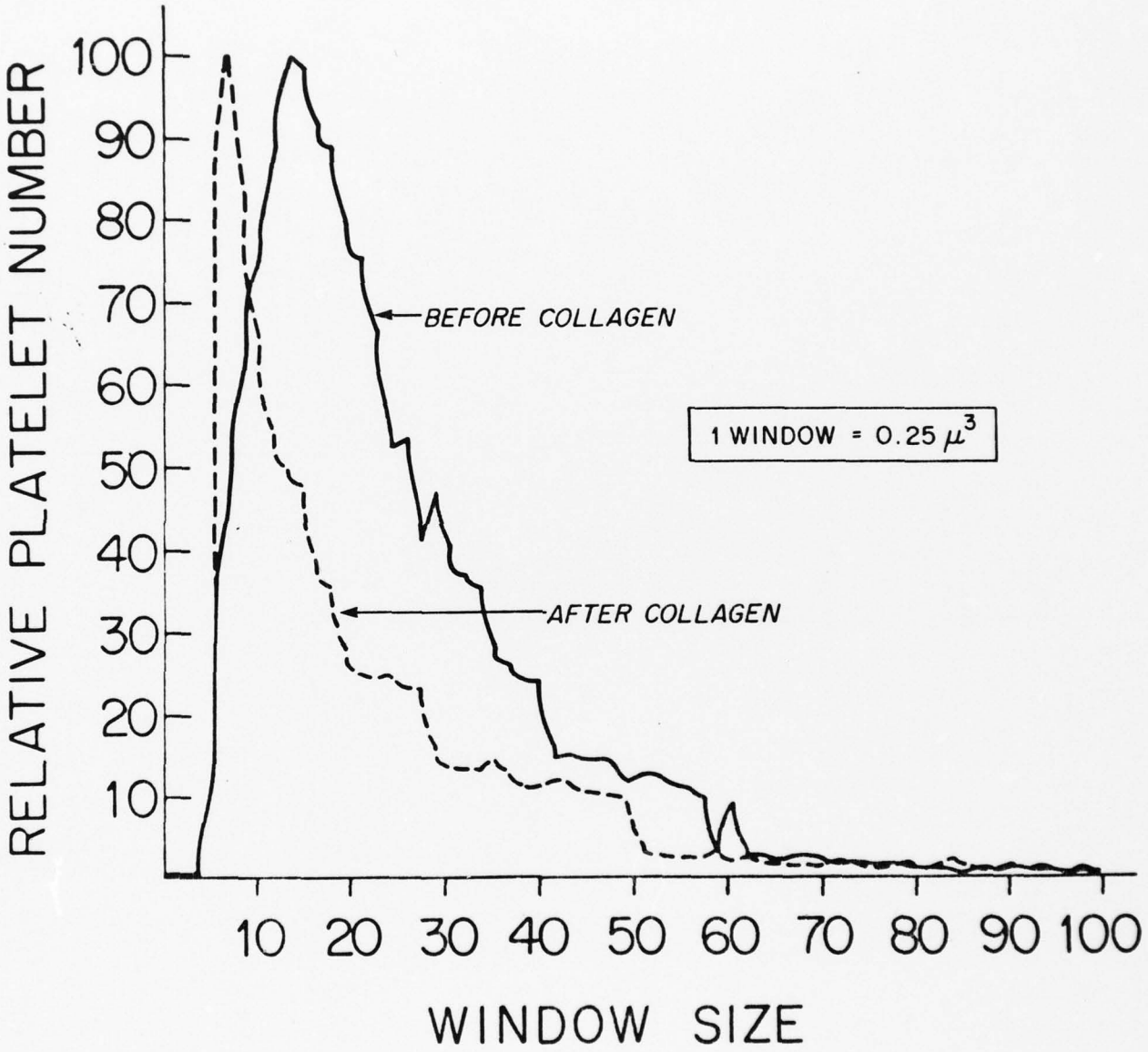
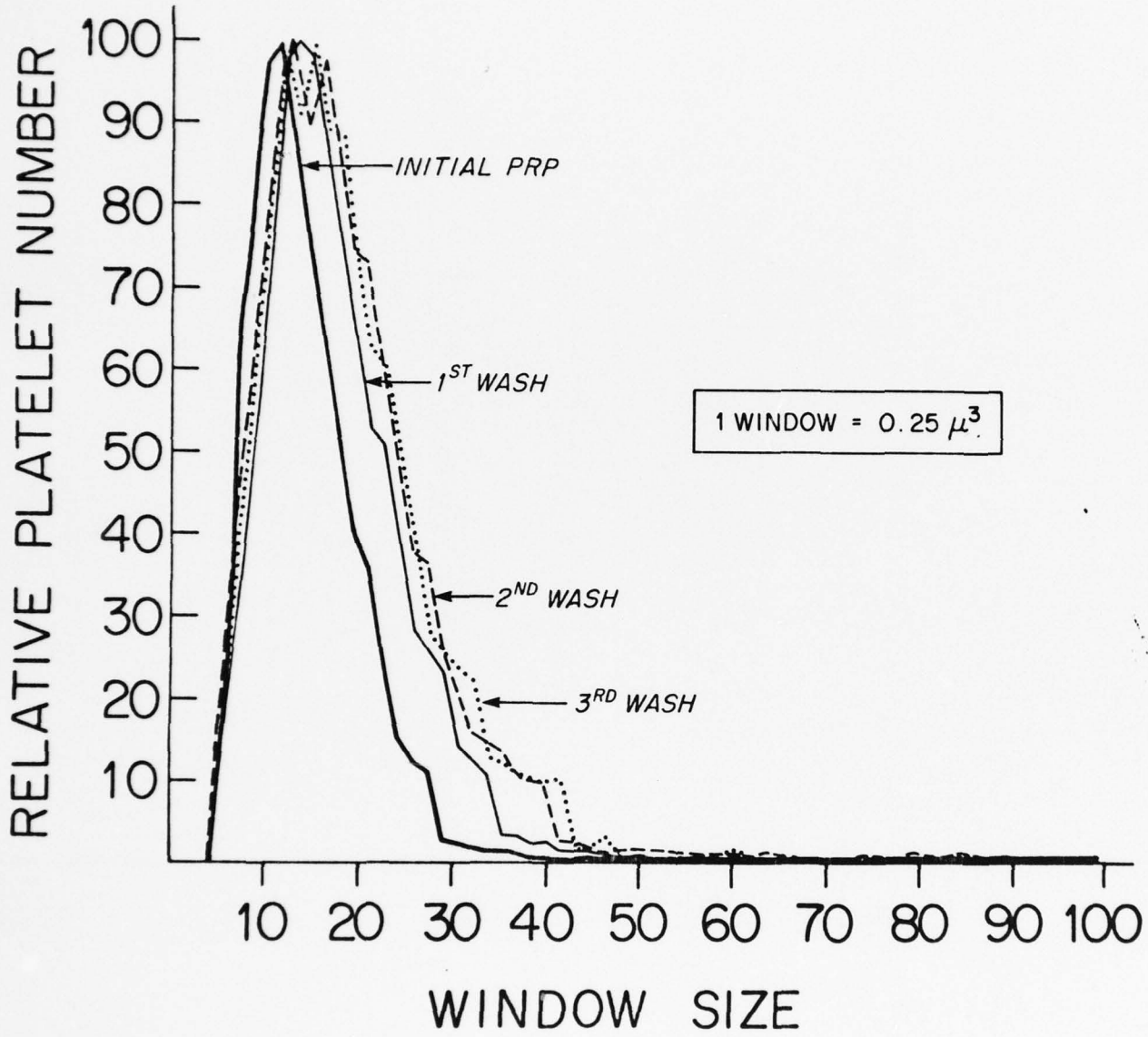


Fig 7.

PLATELET VOLUME DISTRIBUTION



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